Subject /Course	Pharmaceutical Biotechnology
Subject/Course ID	BP605T
Module Title	Study of Cloning Vectors, Recombinant DNA technology, PCR
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Learning Outcome of module-2

LO	Learning Outcome (LO)	Course Outcome Code
LO1	Upon completion of topic, students will be able to understand the concept of cloninig vectors and its applications.	BP605.2
LO2	They will be familiar with Recombinant DNA technology concept and its use in pharmaceutical manufacturing.	BP605.2
LO3	They will have knowledge about genetic engineering and its uses.	BP605.2
LO4	They shall be able to understand the concept of PCR	BP605.2

MODULE CONTENT TABLE

Topic
• Study of cloning vectors • Study of restriction endonucleasesand DNA ligase • Recombinant DNA technology • Application of rDNA technology and genetic engineering in the production of Interferon • Application of rDNA technology and genetic engineering in the production of: Vaccines- hepatitis- B, Hormones-Insulin. • Application of genetic engineering in medicine • Brief introduction to PCR

CLONING VECTORS

A cloning vector is a small piece of DNA that can be stably maintained in an organism, and into which a foreign DNA fragment can be inserted for cloning purposes. The cloning vector may be DNA taken from a virus, the cell of a higher organism, or it may be the plasmid of a bacterium. Cloning vectors provide a backbone for the DNA insert to be reproduced and propagated in bacteria; however, these vectors are only useful for storing a genetic sequence. By themselves, they are incapable of allowing for transcription and translation of the gene into a functional protein product.

Features of Cloning Vectors

The cloning vectors possess the following features:

1) A cloning vector should possess an origin of replication so that it can self-replicate inside the host cell.
2) It should have a restriction site for the insertion of the target DNA.
3) It should have a selectable marker with an antibiotic resistance gene that facilitates screening of the recombinant organism.
4) It should be small in size so that it can easily integrate into the host cell.
5) It should be capable of inserting a large segment of DNA.
6) It should possess multiple cloning sites.
7) It should be capable of working under the prokaryotic and eukaryotic systems.

Types of Cloning Vectors

There are the following different types of cloning vectors:

Plasmids

These were the first vectors used in gene cloning.

These are found in bacteria, eukaryotes and archaea.

These are natural, extrachromosomal, self-replicating DNA molecules.

They have a high copy number and possess antibiotic-resistant genes.

They encode proteins which are necessary for their own replication.

pBR322, pUC18, F-plasmid are some of the examples of plasmid vectors

Bacteriophage

These are more efficient than plasmids for cloning large DNA inserts.

Phage λ and M13 phage are commonly used bacteriophages in gene cloning.

53 kb DNA can be packaged in the bacteriophage.

The screening of phage plaques is much easier than the screening of recombinant bacterial colonies.

Phasemids

These are artificial vectors.

They are used in combination with M13 phage.

They possess multiple cloning sites and an inducible lac gene promoter.

They are identified by blue-white screening.

Bacterial Artificial Chromosomes

These are similar to E.coli plasmids vectors.

It is obtained from naturally occurring F' plasmid.

These are used to study genetic disorders.

They can accommodate large DNA sequences without any risk.

Other cloning vectors include:

Yeast Artificial Chromosomes

Cosmids

Retroviral Vectors

Human Artificial Chromosomes

Restriction Endonucleases

Restriction endonucleases (REs) are bacterial enzymes that cleave double-stranded DNA. Type I REs are important in bacterial function but do not cleave DNA at specific sequences. Type II REs, described for use in this manual, require highly specific sites for DNA cleavage and are thus extremely useful tools in molecular biology. These enzymes allow the cloning and purification of defined DNA fragments. The 500 or so known REs are typically isolated from a variety of bacterial strains. REs are present in bacteria presumably to destroy DNA from foreign sources (e.g., infecting bacteriophage) by cleaving the foreign DNA at specific recognition sites.

The host bacteria's DNA is protected from cleavage because the specific recognition sites are modified, usually by methylation at one of the bases in the site, making the site no longer a substrate for RE cleavage. Practically, REs with different recognition site specificities have been purified from various bacterial strains and are used by molecular biologists under defined conditions to cleave purified DNA from eukaryotic sources into defined fragments in an in vitro reaction. Host bacteria used to propagate cloned DNA in the laboratory are usually mutant in the host restriction genes (hsdR, hsdM, or hsdS); thus their intracellular enzyme activities will not destroy the foreign recombinant sequences.

DNA Ligase

DNA ligase IV has an extended C-terminal region that contains tandemly arrayed BRCT motifs (Figure 2). The linker region between the two BRCT motifs mediates an interaction with the DNA repair protein XRCC4 that is required for the stability and activity of DNA ligase IV. DNA ligase IV stability is also influenced by phosphorylation of Ser650 by the DNA-dependent protein kinase (DNA-PK). Unlike DNA ligases I and III, the majority of DNA ligase IV molecules in the DNA ligase IV/XRCC4 complex, after purification, are pre-adenylated and only catalyze a single ligation event. However, the DNA repair protein XLF, which interacts with XRCC4, stimulates the joining of compatible and incompatible DNA ends by DNA ligase IV/XRCC4. DNA ligases catalyse the formation of a phosphodiester bond between adjacent 3′ hydroxyl and 5′ phosphate terminal groups in double stranded DNA. Their primary biological role is to seal single stranded “nicks” in double stranded DNA, which arise from a diversity of cellular processes including DNA replication (Ogawa & Okazaki, 1980) and repair (Timson, Singleton, &Wigley, 2000). However, DNA ligases also have numerous applications in molecular biology and biotechnology owing to their utility in generating recombinant DNA.

Recombinant DNA Technology

Recombinant DNA, molecules of DNA from two different species that are inserted into a host organism to produce new genetic combinations that are of value to science, medicine, agriculture, and industry. Since the focus of all genetics is the gene, the fundamental goal of laboratory geneticists is to isolate, characterize, and manipulate genes. Although it is relatively easy to isolate a sample of DNA from a collection of cells, finding a specific gene within this DNA sample can be compared to finding a needle in a haystack. Consider the fact that each human cell contains approximately 2 metres (6 feet) of DNA. Therefore, a small tissue sample will contain many kilometres of DNA. However, recombinant DNA technology has made it possible to isolate one gene or any other segment of DNA, enabling researchers to determine its nucleotide sequence, study its transcripts, mutate it in highly specific ways, and reinsert the modified sequence into a living organism.

Isolation of Genetic Material

The first step in rDNA technology is to isolate the desired DNA in its pure form i.e. free from other macromolecules. Since DNA exists within the cell membrane along with other macromolecules such as RNA, polysaccharides, proteins, and lipids, it must be separated and purified which involves enzymes such as lysozymes, cellulase, chitinase, ribonuclease, proteases etc. Other macromolecules are removable with other enzymes or treatments. Ultimately, the addition of ethanol causes the DNA to precipitate out as fine threads. This is then spooled out to give purified DNA.

Restriction Enzyme Digestion

Restriction enzymes act as molecular scissors that cut DNA at specific locations. These reactions are called 'restriction enzyme digestions'. They involve the incubation of the purified DNA with the selected restriction enzyme, at conditions optimal for that specific enzyme. The technique 'Agarose Gel Electrophoresis' reveals the progress of the restriction enzyme digestion. This technique involves running out the DNA on an agarose gel. On the application of current, the negatively charged DNA travels to the positive electrode and is separated out based on size. This allows separating and cutting out the digested DNA fragments. The vector DNA is also processed using the same procedure.

Amplification Using PCR

Polymerase Chain Reaction or PCR is a method of making multiple copies of a DNA sequence using the enzyme – DNA polymerase in vitro.It helps to amplify a single copy or a few copies of DNA into thousands to millions of copies.

Ligation of DNA Molecules

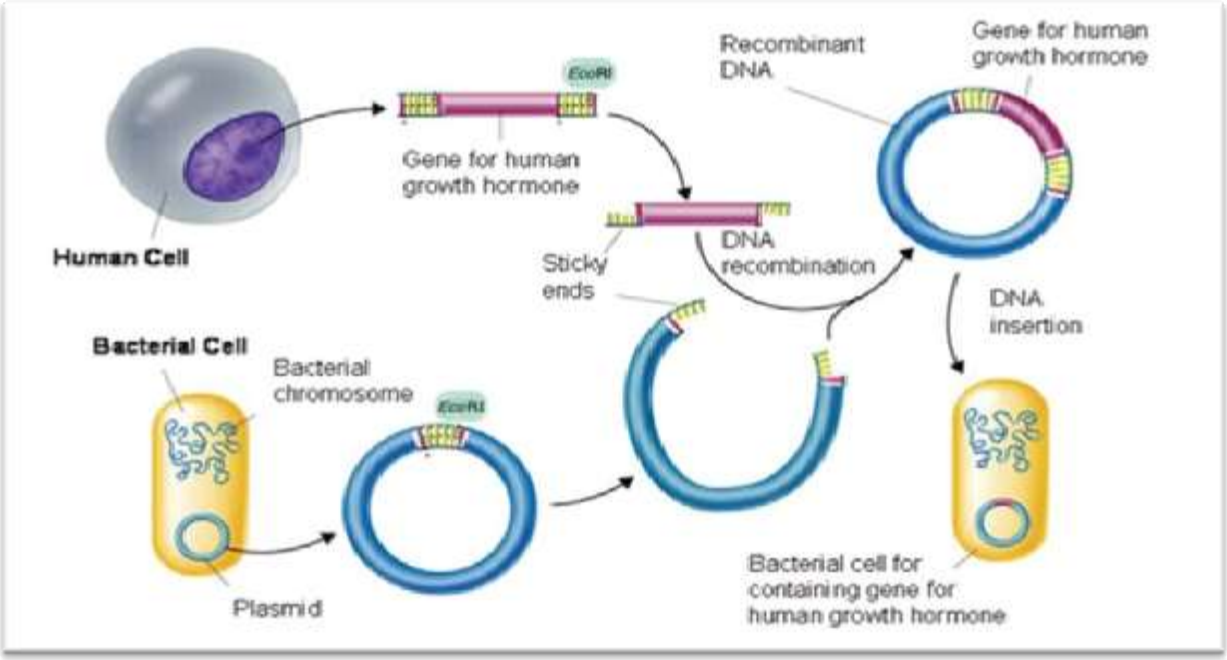
The purified DNA and the vector of interest are cut with the same restriction enzyme. This gives us the cut fragment of DNA and the cut vector, that is now open. The process of joining these two pieces together using the enzyme 'DNA ligase' is 'ligation'. The result-ing DNA molecule is a hybrid of two DNA molecules – the interest molecule and the vector. In the ter-minology of genetics this intermixing of dif-ferent DNA strands is called recombination. Hence, this new hybrid DNA molecule is also called a recombinant DNA molecule and the technology is referred to as the recom-binant DNA technology.

Insertion of Recombinant DNA into Host

In this step, the recombinant DNA is introduced into a recipient host cell mostly, a bacterial cell. This process is 'Transformation'. Bacterial cells do not accept foreign DNA easily. Therefore, they are treated to make them 'competent' to accept new DNA. The processes used may be thermal shock, Ca++ ion treatment, electroporation etc.

Isolation of Recombinant Cells

The transformation process generates a mixed population of transformed and non-trans- formed host cells. The selection process involves filtering the transformed host cells only. For isolation of recombinant cell from non-recombinant cell, marker gene of plasmid vector is employed. For examples, PBR322 plasmid vector contains different marker gene (Ampicillin resistant gene and Tetracycline resistant gene. When pst1 RE is used it knock out Ampicillin resistant gene from the plasmid, so that the recombinant cell become sensitive to Ampicillin.



Interferons

(IFNs, /ˌɪntərˈfɪərɒn/[1]) are a group of signaling proteins made and released by host cells in response to the presence of several viruses. In a typical scenario, a virus-infected cell will release interferons causing nearby cells to heighten their anti-viral defenses. IFNs belong to the large class of proteins known as cytokines, molecules used for communication between cells to trigger the protective defenses of the immune system that help eradicate pathogens Interferons are named for their ability to "interfere" with viral replication by protecting cells from virus infections. However, virus-encoded genetic elements has the ability to antagonize the IFN response contributing to viral pathogenesis and viral diseases. IFNs also have various other functions: they activate immune cells, such as natural killer cells and macrophages; they increase host defenses by up-regulating antigen presentation by virtue of increasing the expression of major histocompatibility complex (MHC) antigens. Certain symptoms of infections, such as fever, muscle pain and "flu-like symptoms", are also caused by the production of IFNs and other cytokines. More than twenty distinct IFN genes and proteins have been identified in animals, including humans. They are typically divided among three classes: Type I IFN, Type II IFN, and Type III IFN. IFNs belonging to all three classes are important for fighting viral infections and for the regulation of the immune system.

Interferon type I: All type I IFNs bind to a specific cell surface receptor complex known as the IFN-α/β receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains. The type I interferons present in humans are IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω. In general, type I interferons are produced when

the body recognizes a virus that has invaded it. They are produced by fibroblasts and monocytes. However, the production of type I IFN-α is inhibited by another cytokine known as Interleukin-10. Once released, type I interferons bind to specific receptors on target cells, which leads to expression of proteins that will prevent the virus from producing and replicating its RNA and DNA. Overall, IFN-α can be used to treat hepatitis B and C infections, while IFN-β can be used to treat multiple sclerosis.

Interferon type II (IFN-γ in humans): This is also known as immune interferon and is activated by Interleukin-12.Type II interferons are also released by cytotoxic T cells and type-1 T helper cells. However, they block the proliferation of type-2 T helper cells. The previous results in an inhibition of Th2 immune response and a further induction of Th1 immune response.[8] IFN type II binds to IFNGR, which consists of IFNGR1 and IFNGR2 chains.

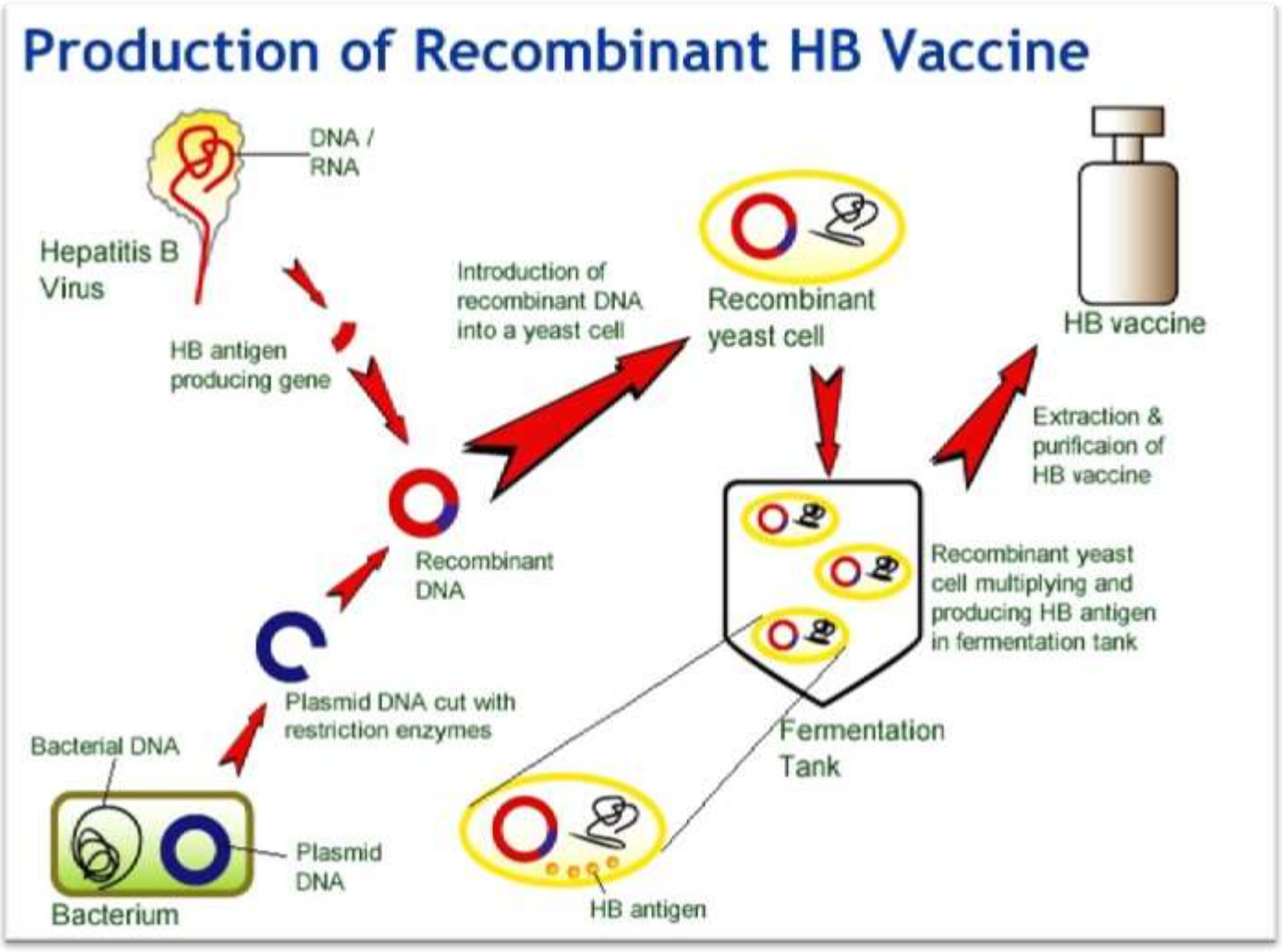
Interferon type III: Signal through a receptor complex consisting of IL10R2 (also called CRF2-4) and IFNLR1 (also called CRF2-12). Although discovered more recently than type I and type II IFNs,[9] recent information demonstrates the importance of Type III IFNs in some types of virus or fungal infections.

Hepatitis B

It is a vaccine-preventable liver infection caused by the hepatitis B virus (HBV). Hepatitis B is spread when blood, semen, or other body fluids from a person infected with the virus enters the body of someone who is not infected.

Signs and symptoms of hepatitis B range from mild to severe. They usually appear about one to four months after you've been infected, although you could see them as early as two weeks post-infection. Some people, usually young children, may not have any symptoms. Hepatitis B signs and symptoms may include:

1) Abdominal pain
2) Dark urine
3) Fever
4) Joint pain
5) Loss of appetite
6) Nausea and vomiting
7) Weakness and fatigue
8) Yellowing of your skin and the whites of your eyes (jaundice)



Causes

Hepatitis B infection is caused by the hepatitis B virus (HBV). The virus is passed from person to person through blood, semen or other body fluids. It does not spread by sneezing or coughing. Common ways that HBV can spread are:

Sexual contact: You may get hepatitis B if you have unprotected sex with someone who is infected. The virus can pass to you if the person's blood, saliva, semen or vaginal secretions enter your body.

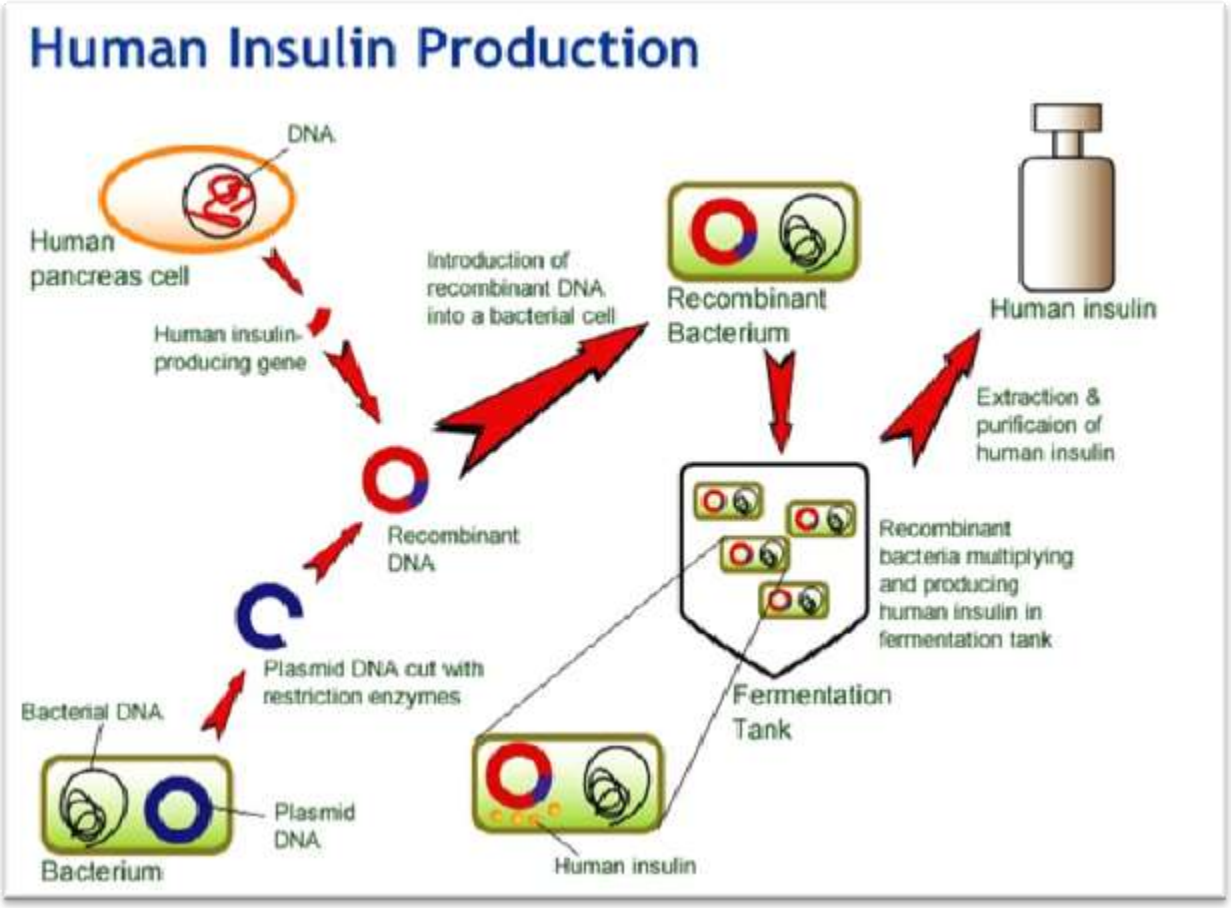
Sharing of needles: HBV easily spreads through needles and syringes contaminated with infected blood. Sharing IV drug paraphernalia puts you at high risk of hepatitis B.

Accidental needle sticks: Hepatitis B is a concern for health care workers and anyone else who comes in contact with human blood.

Mother to child: Pregnant women infected with HBV can pass the virus to their babies during childbirth. However, the newborn can be vaccinated to avoid getting infected in almost all cases. Talk to your doctor about being tested for hepatitis B if you are pregnant or want to become pregnant.

INSULIN

Insulin is a hormone created by your pancreas that controls the amount of glucose in your bloodstream at any given moment. It also helps store glucose in your liver, fat, and muscles. Finally, it regulates your body's metabolism of carbohydrates, fats, and proteins. Sound important? That's because it is."Without proper insulin function, your body can't store glucose in your muscles or liver, but neither can it make any fat. Instead, the fat breaks down and produces, among other things, keto acids," says endocrinologist Irl Hirsh, MD. If the levels of these acids grow too high, the imbalance can trigger diabetic ketoacidosis, a potentially fatal condition. When you eat, your blood glucose levels rise, and this leads a typical person's pancreas to release insulin, so that the sugar can be stored as energy for later use. Without that pancreatic ability, as a person with either type 1 diabetes or advanced type 2 diabetes, your blood sugar levels may rise dangerously high, or drop too low.



Different types of Insulin

Onset is defined as the length of time before insulin hits your bloodstream and begins to lower blood glucose.

Peak is the time during which insulin is at its maximum effectiveness at lowering your blood glucose levels.

Duration is the length of time insulin continues to lower your blood glucose levels.

POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR) is a method widely used to rapidly make millions to billions of copies (complete copies or partial copies) of a specific DNA sample, allowing scientists to take a very small sample of DNA and amplify it (or a part of it) to a large enough amount to study in detail. PCR was invented in 1983 by the American biochemist Kary Mullis at Cetus Corporation. It is fundamental to many of the procedures used in genetic testing and research, including analysis of ancient samples of DNA and identification of infectious agents. Using PCR, copies of very small amounts of DNA sequences are exponentially amplified in a series of cycles of temperature changes. PCR is now a common and often indispensable technique used in medical laboratory research for a broad variety of applications including biomedical research and criminal forensics. The majority of PCR methods rely on thermal cycling. Thermal cycling exposes reactants to repeated cycles of heating and cooling to permit different temperature-dependent reactions—specifically, DNA melting and enzyme-driven DNA replication. PCR employs two main reagents—primers (which are short single strand DNA fragments known as oligonucleotides that are a complementary sequence to the target DNA region) and a DNA polymerase. In the first step of PCR, the two strands of the DNA double helix are physically separated at a high temperature in a process called nucleic acid denaturation. In the second step, the temperature is lowered and the primers bind to the complementary sequences of DNA. The two DNA strands then become templates for DNA polymerase to enzymatically assemble a new DNA strand from free nucleotides, the building blocks of DNA. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the original DNA template is exponentially amplified.

PCR amplifies a specific region of a DNA strand (the DNA target). Most PCR methods amplify DNA fragments of between 0.1 and 10 kilo base pairs (kbp) in length, although some techniques allow for amplification of fragments up to 40 kbp. The amount of amplified product is determined by the

available substrates in the reaction, which becomes limiting as the reaction progresses. A basic PCR set-up requires several components and reagents,[7] including:

DNA template that contains the DNA target region to amplify

DNA polymerase; an enzyme that polymerizes new DNA strands; heat-resistant Taq polymerase is especially common,[8] as it is more likely to remain intact during the high-temperature DNA denaturation process two DNA primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strands of the DNA target (DNA polymerase can only bind to and elongate from a double-stranded region of DNA; without primers, there is no double-stranded initiation site at which the polymerase can bind);[9] specific primers that are complementary to the DNA target region are selected beforehand, and are often custom-made in a laboratory or purchased from commercial biochemical suppliers deoxynucleoside triphosphates, or dNTPs (sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups), the building blocks from which the DNA polymerase synthesizes a new DNA strand

a buffer solution providing a suitable chemical environment for optimum activity and stability of the DNA polymerase bivalent cations, typically magnesium (Mg) or manganese (Mn) ions; Mg2+ is the most common, but Mn2+ can be used for PCR-mediated DNA mutagenesis, as a higher Mn2+ concentration increases the error rate during DNA synthesis;[10] and monovalent cations, typically potassium (K) ions.

QUESTION BANK

2 Marks Questions

1. Define PCR.
2. What are DNA Ligases and restriction endonucleases?
3. Define Cloning Vectors.
4. Define Genetic Engineering.
5. What is the role of genetic engineering in medical field?
6. What are Interferon?
7. What is Differential display PCR?
8. Define Quantitative real time PCR.
9. Define Vaccines.
10. Define Gene mapping and Chromosomal mapping.

5 Marks Questions

1. Enlist the steps involved in the rDNA technology.
2. Write a brief note on PCR.
3. Explain the applications genetic engineering in medicine.
4. Explain the applications of rDNAteccnology.

10 Marks Questions

1. Write a note on Cloning Vectors.
2. Write a detail note on production of Hepatitis B and Insulin.
3. Explain various steps used in Polymerase Chain Reaction.
4. Explain the applications of Restriction Endonucleases and DNA Ligase.